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User Manual

PathHunter® IL-2 Bioassay Kit (Dimerization)

For Detection of Ligand-Induced Dimerization of IL-2 Receptor Subunits

For Bioassay Kits with ligand:

93-1003Y3-00091: 2-Plate Kit

93-1003Y3-00092: 10-Plate Kit

For Bioassay Kit without ligand

93-1003Y3-00187: 10-Plate Kit

Document Number 70-386 Rev 1

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Important: Please read this entire user manual before proceeding with the assay.

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1. Overview

The PathHunter IL-2 Bioassay Kit (Dimerization) is a simple, robust, non-radioactive, dye free assay for quantitation of IL-2 mediated dimerization of the IL-2 receptor (IL2Rβ/IL2Rγ/IL2Rα). The PathHunter IL-2 bioassay kit with ligand contains all the materials needed to perform a complete assay, including cryopreserved, single-use cells, detection reagents, cell plating reagent, agonist for stimulating the cells, and assay plates. A 10-Plate PathHunter IL-2 Bioassay Kit is also offered without ligand but contains all other components listed above to run the assay. This bioassay has been optimized for a 96-well plate format.

2. Assay Principle

This bioassay utilizes Enzyme Fragment Complementation (EFC) technology, where the β-galactosidase (β-gal) enzyme is split into two fragments, the ProLink™ (PK), and the Enzyme Acceptor (EA). Independently, these fragments have no β-gal enzymatic activity, however, when forced to complement through protein-protein interactions, they form an active β-gal enzyme.

The PathHunter IL-2 Bioassay evaluates activity in the IL2Rβ/IL2Rɣ/IL2Rα Dimerization assay, an application of the Eurofins DiscoverX Dimerization Assay platform. The assay is designed to detect the ligand-induced heterodimerization of the IL2Rβ, IL2Rɣ and IL2Rα receptors, which comprise the high affinity receptor for IL-2. As shown in Figure 1, the bioassay cells have been engineered to co-express IL2Rβ fused to PK, and IL2Rɣ fused to EA. Binding of IL-2 to IL2Rα causes dimerization of the receptor chains, which brings the two β-gal fragments (PK and EA) into close proximity, forcing complementation. The result is formation of a functional β-gal enzyme that hydrolyzes the substrate to generate a chemiluminescent signal.

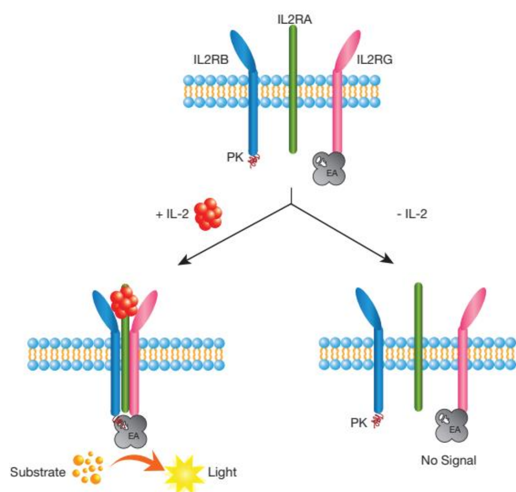


Figure 1. PathHunter IL-2 Bioassay (Dimerization) Assay Principle.

3. Materials Provided in PathHunter IL-2 Bioassay Kit (Dimerization)

List of Components	93-1003Y3-00091 (2-Plate Kit)	93-1003Y3-00092 (10-Plate Kit)	93-1003Y3-00187 (10-Plate Kit without ligand)
PathHunter U2OS IL2B/IL2RG/IL2RA Bioassay Cells (0.6 x 10^6 cells in 0.1 mL per vial)	2	10	10
AssayComplete™ Cell Plating 5 Reagent (100 mL per bottle)	1 x 100 mL	3 x 100 mL	3 x 100 mL
AssayComplete Protein Dilution Buffer (Bottle)	1 x 50 mL	2 x 50 mL	2 x 50 mL
Recombinant Human IL-2 (10 µg per vial)	1	1	N/A*
PathHunter Bioassay Detection Kit Detection Reagent 1 (Bottle) Detection Reagent 2 (Bottle)	 1 x 3 mL 1 x 12 mL	 1 x 15 mL 1 x 60 mL	 1 x 15 mL 1 x 60 mL
96-Well White, Clear Flat-Bottom, TC-Treated, Sterile Plates with Lid	2 Plates	10 Plates	10 Plates

Note * for 93-1003Y3-00187 ligand not provided in the kit, would need to be obtained separately if needed

4. Storage Conditions

PathHunter U2OS IL2RB/IL2RG/IL2RA Bioassay Cells

Cells are shipped on dry ice and should arrive in a frozen state. To ensure maximum cell viability, store the vials of bioassay cells in the vapor phase of liquid nitrogen as soon as possible upon receipt. Please contact technical support immediately, if the cells received were already thawed.

- Short-term (24 hours or less): Store vials at -80°C immediately upon arrival. (DO NOT store at -80°C for longer than 24 hours).
- Long-term (greater than 24 hours): Vials should ONLY be stored in the vapor phase of liquid nitrogen.

Safety Warning: A face shield, gloves and lab coat should be worn at all times when handling frozen vials. Use tongs to remove cryovials from liquid nitrogen storage, and place the vials immediately in dry ice in a covered container. Wait for at least 1 minute for any liquid nitrogen that may be present inside the vial to evaporate. Do not touch the bottom of the vials at any time to avoid inadvertent thawing of the cells.

AssayComplete™ Cell Plating 5 (CP5) Reagent

Upon receipt, store at -20°C. Once thawed, the Cell Plating Reagent can be stored at 4°C for up to 4 weeks. For longer storage (up to the expiration date listed in the kit's Certificate of Analysis), aliquot the reagent and store at -20°C until needed. Do not freeze-thaw more than three times.

To make aliquots suitable for testing one assay plate each, 30 mL of reagent per aliquot can be dispensed and frozen down.

AssayComplete™ Protein Dilution Buffer

Once thawed, Protein Dilution Buffer can be stored at 4°C for up to 4 weeks. For longer storage (up to the expiration date listed in the kit’s Certificate of Analysis), the reagent should be aliquoted and stored at -20°C until needed. Avoid multiple freeze-thaw cycles. To make aliquots suitable for testing one assay plate each,

10 mL of reagent per aliquot should be dispensed and frozen down. This amount may vary depending on the stock sample concentrations, and should be adjusted accordingly.

Recombinant Human IL-2 (If supplied in the kit)

Upon receipt, store at -20°C, until ready to use (up to the date listed in the kit's Certificate of Analysis). Centrifuge the vial before opening to maximize recovery. Reconstitute to a concentration of 100 µg/mL by adding 100 µL of supplied reconstitution buffer to the 10 µg vial. Once prepared, the stock solution should be stored as suitable aliquots (e.g. 30 µL) at -20°C until needed. Do not freeze/thaw more than twice. Reconstituted ligand is stable for 12 months at -20°C to -80°C, or 1 week at 2-8°C.

PathHunter Bioassay Detection Kit

Upon receipt, store at -20°C. Once thawed, the detection reagents can be kept at 4°C for up to 4 days. For long-term storage (up to the expiration date listed in the kit's Certificate of Analysis), the reagent should be aliquoted and stored at -20°C until needed. Avoid multiple freeze-thaw cycles.

For the ten-plate kit: If all the plates will not be used at the same time, we recommend making five aliquots for each of the two detection reagents. Each aliquot will be adequate for two assay plates. Make five aliquots of 3 mL each for Detection Reagent 1, and five aliquots of 12 mL each for Detection Reagent 2. Sufficient reagent volumes are provided in the kit to make these aliquots.

If the reagents will be used for a single plate, then the remaining detection reagents can be frozen. The detection reagents can be thawed and frozen for a total of three times without loss in performance.

96-Well Tissue Culture-Treated Plates

Store at room temperature.

5. Additional Materials and Equipment Recommended for Assay

The following equipment and additional materials are required to perform these assays:

Materials	Ordering Information
Recombinant Human IL-2	DiscoverX (92-1253), or similar
96-Well Green, V-Bottom, Untreated, Non-Sterile Dilution Plates	DiscoverX (92-0011), or similar
Multimode or luminescence plate reader	Refer to Instrument Compatibility Chart at discoverx.com/instrument-compatibility
Disposable reagent reservoir	Thermo Fisher Scientific, Cat. No. 8094 or similar
Humidified tissue culture incubator (37°C and 5% CO_2)	
Single and multichannel micropipettes and pipet tips	
50 mL and 15 mL polypropylene tubes	
1.5 mL microcentrifuge tubes	
Single and multichannel pipettors (e.g. P20, P100, P1000)	

6. Unpacking Cell Cryovials

Cryovials are shipped on dry ice and contain cells in 0.1 mL of AssayComplete™ Freezing Reagent (refer to the CoA for cell number provided in the vial). Upon receipt, the vials should be transferred to the vapor phase of liquid nitrogen for storage beyond 24 hours. The following procedures are for the safe storage and removal of cryovials from liquid nitrogen storage.

Appropriate personal protective equipment (PPE), including a face shield, gloves, and a lab coat should be worn during these procedures.

PathHunter U2OS IL2B/IL2RG/IL2RA Bioassay Cells must arrive in a frozen state on dry ice.

Contact technical support immediately, if cells received were already thawed.

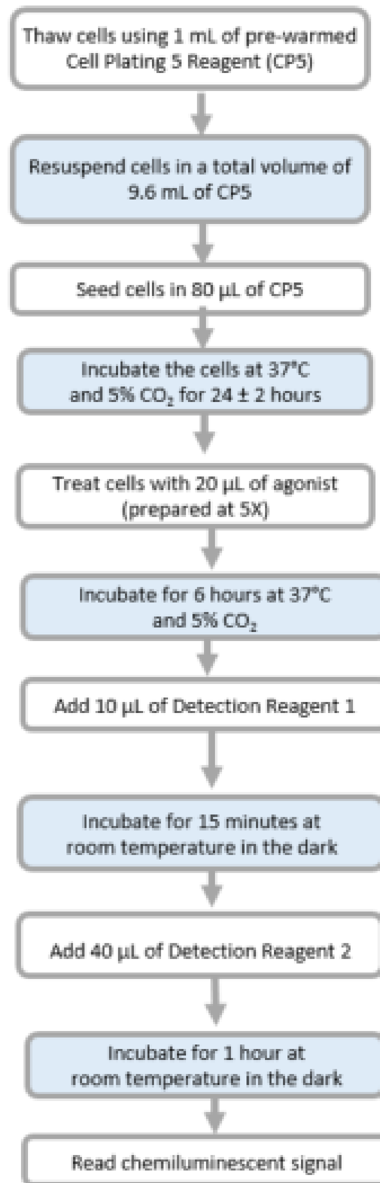
1. Frozen cells must be transferred to either liquid nitrogen storage or a -80°C freezer immediately upon arrival. If cells will be thawed and used within 24 hours, they can be stored temporarily at -80°C. For long-term storage, place vials in the vapor phase of liquid nitrogen storage.

Cryovials should be stored in the vapor phase of liquid nitrogen ONLY. These vials are not rated for storage in the liquid phase.

2. Using tongs, remove the cryovials from the liquid nitrogen storage, and place them immediately in dry ice, in a covered container. Wait for at least one minute for any liquid nitrogen inside the vial to evaporate.
3. Proceed with the thawing protocol in the detailed assay protocol section. Refer to the CoA for the appropriate AssayComplete products mentioned in the protocol below.

7. Protocol Schematic

Quick-Start Procedure: In a 96-well tissue culture-treated plate, perform the following steps.



*Room temperature refers to a range of 23-25°C

8. Detailed Assay Protocol

This user manual provides a protocol for quantifying IL-2 mediated dimerization of the receptor for IL-2 (IL2Rβ/IL2Rγ/IL2Rα). This assay is performed under aseptic conditions. Preparation of cells, Reference Standards/Test Samples are performed in a Biological Safety Cabinet following good aseptic technique.

All appropriate materials are either certified sterile or prepared aseptically.

If purchasing the bioassay kit without ligand, IL-2 ligand can be sourced per the details in the Additional Materials and Equipment Recommended for Assay table.

8.1 Bioassay Cell Preparation

Day 1:

The following protocol is for thawing and plating frozen PathHunter U2OS IL2RB/IL2RG/IL2RA Bioassay Cells from cryovials.

1. Prior to thawing the cells, ensure that all the required materials are set up in the tissue culture hood. These include:

    a. One 25 mL reagent reservoir.

    b. One 15 mL conical tube.

    c. A micropipette (P1000) set to dispense 1 mL.

    d. A multichannel pipette and tips set to dispense 80 µL.

    e. A bottle of Cell Plating Reagent 5 (CP5, pre-warmed in a 37˚C water bath for 15 minutes).

    f. A white-walled, clear-bottom 96-well assay plate.

2. Dispense 9.6 mL of CP5 into the 15 mL conical tube.

DO NOT use heated water bath to thaw the vial. Wipe down the cryovial quickly with 70% ethanol and bring it into the tissue culture hood right away. DO NOT touch the sides or bottom of the vial to avoid thawing of the cell pellet through body heat.

3. Remove the cryovial from the liquid nitrogen vapor and immediately place it in dry ice.

4. Thaw the pellet by immediately adding 1 mL (using P1000) of pre-warmed CP5 from the 15 mL conical tube to the cryovial, thawing the cell pellet. Add 1 mL of pre-warmed CP5 from the 15 mL conical tube, to the cryovial to thaw the cell pellet. The reagent should be added slowly along the wall of the cryovial tube. Slowly pipet up and down 3 times to uniformly suspend the cells.

5. Transfer the cell suspension to the conical tube containing the remaining 8.6 mL of CP5. Remove all the suspension from the cryovial tube to ensure maximum recovery of all the cells.

6. Replace the cap on the conical tube and mix by gentle inversion 2-3 times to ensure that the cells are properly resuspended in the reagent, without creating any froth in the suspension. Pour it immediately into the sterile 25 mL reservoir.

7. Using a multichannel pipet, transfer 80 µL of the cell suspension to each well of the 96-well assay plate, one row at a time, using reverse pipetting. Mix cells in trough by pipetting up/down 2-3 times before aspirating and pipetting cells into each subsequent row in the assay plate.

8. Replace the lid on assay plate and leave the plate at room temperature in biosafety cabinet for 15 minutes (but no more than 30 minutes) to allow the cells to settle uniformly in the well, to minimize potential for edge effects.

9. Gently place the assay plate in a humidified tissue culture incubator set to 37˚C and 5% CO_2 for 24 ± 2 hours before proceeding.

8.2 Sample Preparation

Day 2

The following protocol is an example for preparing a serial dilution of IL-2 reference control.

10. Prepare the reference agonist, IL-2, dose response curve, which will serve as a positive control in this assay. Agonist is prepared at 5X the desired final concentration as it will be diluted by adding 20 µL to the 80 µL of medium present in the assay plate. The top dose for the Eurofins DiscoverX (part #92-1253) IL-2 control is 100 ng/mL (final concentration).

    a. Add 200 µL of Protein Dilution Buffer (PDB) to wells A2 to A12 of a master dilution plate (e.g. a V-bottom polypropylene 96-well dilution plate, DiscoverX, Cat. No. 92-0011 or similar).

    b. Add 100 µL of supplied Reconstitution Buffer to the IL-2 vial containing 10 µg of lyophilized powder to make a 100 µg/mL stock solution. Gently shake (do not vortex) for ten minutes to increase solubility.

    c. Make a 10 µg/mL intermediate dilution by adding 180 µL of PDB to a non-binding eppendorf tube. Add 20 µL of the 100 µg/mL IL-2 stock to this tube (for a 1:10 dilution). Mix thoroughly by pipetting up and down several times with a pipette tip set to at least 100 µL.

    d. Prepare a 500 ng/mL working solution by adding 25 µL of the 10 µg/mL IL-2 intermediate dilution from step 1c to 475 µL of PDB in a fresh non-binding eppendorf tube. This 500 ng/mL working solution represents 5X the final 100 ng/mL concentration in top dose of DRC.

    e. Transfer 200 µL of the 500 ng/mL IL-2 working solution to well A1 of the master dilution plate.

    f. Using a clean tip, transfer 100 µL of IL-2 from well A1 into well A2 and mix by pipetting up and down several times. Replace the pipette tip, and transfer 100 µL from well A2 into well A3. Mix by pipetting up and down several times. Repeat this process until well A11 is reached, resulting in an eleven point, 1:3 dilution series. No ligand is transferred to column 12 as this will serve as a negative control.

11. Prepare test sample in a similar manner to reference agonist, using a new row of the master dilution plate.

12. Remove the assay plate from the 37°C and 5% CO_2 incubator and bring into the tissue culture hood.

13. Transfer 20 µL of dilution series from each sample prepared in the master dilution plate to the appropriate wells of the assay plate, as shown in the Representative Assay Plate Map (Figure 3)

14. Return the assay plate to the 37°C and 5% CO_2 incubator and incubate for 6 hours before continuing with the detection steps.

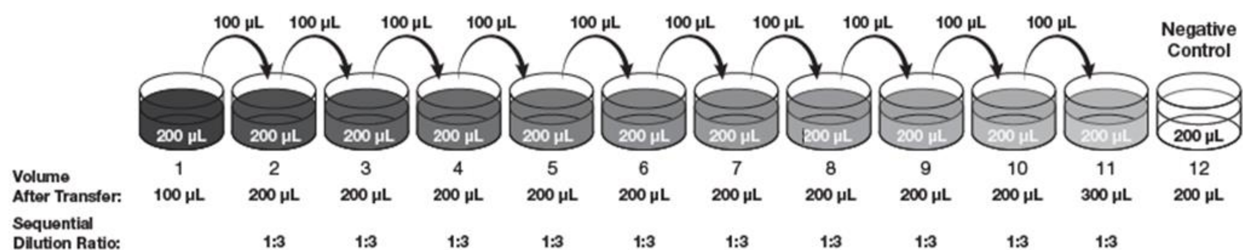


Figure 2. Agonist Dilution Series: Make eleven 3-fold serial dilutions of the agonist in a master dilution plate.

8.3 Addition of Detection Reagent

Day 2: Signal Detection

1. Using a multichannel pipette, add 10 µL of Detection Reagent 1 to each well of the assay plate. Place the plate onto an orbital shaker at 350 rpm for 1 minute to cause even mixing
2. Incubate the plate at room temperature (23-25°C) for 15 minutes in the dark.

Detection reagents are light sensitive, thus incubation in the dark is necessary.

Room temperature refers to a range of 23-25°C.

3. Using a multichannel pipette add 40 µL of Detection Reagent 2 to each well of the assay plate.
4. Incubate the plate at room temperature for 1 hour in the dark.
5. Read the sample on a standard luminescence plate reader at 0.1 to 1 second/well for photomultiplier tube (PMT) readers or 5-10 seconds for an imager. To determine instrument compatibility, visit discoverx.com/instrument-compatibility.
6. Data analysis can be performed using any statistical analysis software such as GraphPad Prism, SoftMax Pro, Gen5, or Microsoft Excel etc.

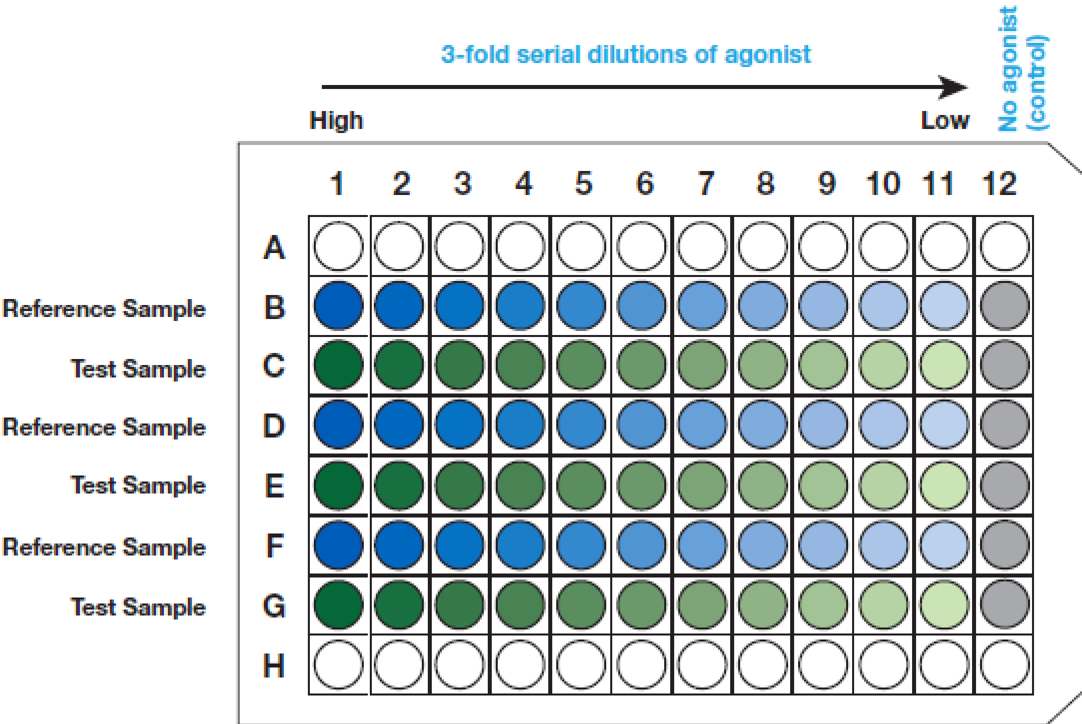


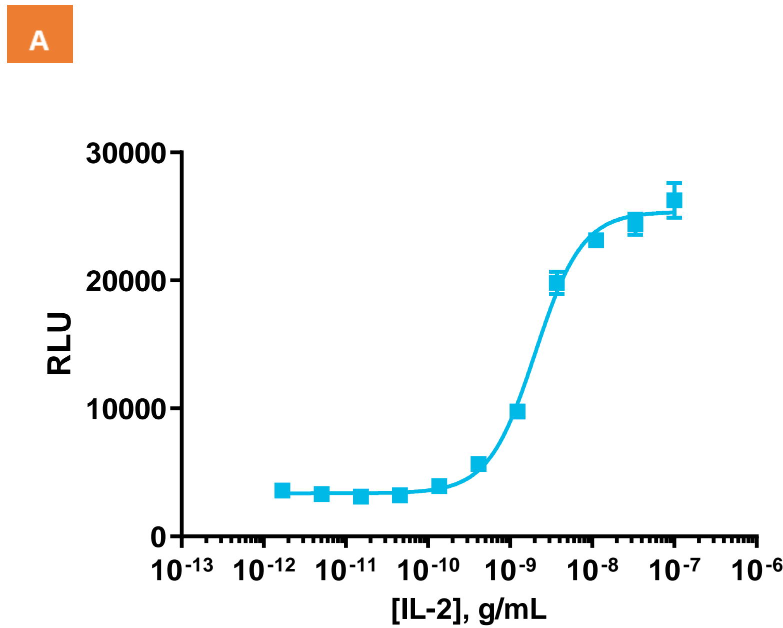
Figure 3. Representative Assay Plate Map: This plate map shows 11-point dilution with 3 data points at each concentration for one reference and one test sample per plate, each prepared with a 1:3 dilution scheme.

9. Typical Results

The following graph is an example of a typical dose-response curve for the PathHunter IL-2 Dimerization Bioassay Kit generated using the protocol outlined in this user manual. The data shows potent, dose-dependent heterodimerization of the IL-2 receptor subunits, when treated with DiscoverX provided IL-2 (Part# 92-1253)

The plate was read on the EnVision® Multimode Plate Reader and data analysis was conducted using GraphPad Prism.

A



B

S/B	EC_{50} (ng/mL)
7.3	2.02

Figure 4. Typical Results:

Representative **A**, dose-response curve and **B**, EC_{50} and assay window for IL-2 mediated dimerization of IL-2 receptor subunits, as measured in this bioassay.

Troubleshooting Guide

Problem	Potential Cause	Proposed Solution
No response	Incorrect thawing procedure	Refer to the thawing instructions in the Bioassay Cell Preparation section of this user manual.
	Incorrect ligand used or incorrect ligand incubation time	Refer to the datasheet for recommended ligand and assay conditions.
	Sub-optimal time course for induction	Optimize time course of induction with the agonist and antagonist.
Low or no signal	Incorrect preparation of detection reagents	Detection reagents are sensitive to light and should ideally be prepared just prior to use.
	Problem with microplate reader	The microplate reader should be in luminescence mode. Read at 0.1-1 second/well.
Experimental S/B does not match the value noted in the Certificate of Analysis	Incorrect incubation temperature	Confirm assay conditions.
		Check and repeat the assay at the correct incubation temperature, as indicated on the assay datasheet.
	Incorrect preparation of ligand (agonist or antagonist)	Some ligands are difficult to handle. Confirm the final concentration of ligands.
	Sub-optimal agonist challenge concentration	Perform the agonist curve to reassess EC_{80} with the ligand provided in the kit. Perform antibody titrations with EC_{80} and EC_{90} agonist challenge concentrations to re-optimize the assay window.
EC_{50} is right-shifted	Improper ligand handling or storage	Check the ligand handling requirements.
	Difference in agonist binding affinity	Refer to the Certificate of Analysis for the ligand provided in the kit to confirm that the ligand used is comparable.
	Problems with plate type	Hydrophobic compounds should be tested for solubility and may require evaluation in different dilution buffers.
	Problems with compound stability	Non-binding surface plates may be necessary for hydrophobic compounds.

For questions on using this product, please contact Technical Support at 1.866.448.4864 or DRX_Support@eurofinsUS.com.

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